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Distinct Genotypes of a Nonenveloped DNA Virus Associated With Posttransfusion Non-A to G Hepatitis (TT Virus) in Plasma and Peripheral Blood Mononuclear Cells

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TT virus (TTV) is a nonenveloped, single-stranded DNA virus with little sequence homology to known viruses, and associated with elevated transaminase levels in the patients with posttransfusion hepatitis of unknown etiology. The DNA of TTV was detected, by semi-nested polymerase chain reaction, in peripheral blood mononuclear cells (PBMC) from the 30 healthy individuals with circulating virus in plasma. A sequence of 222 bases was determined on 6–10 TTV DNA clones each from plasma and 6 clones each from PBMC from eight individuals selected at random from this group. TTV can be classified into genotypes separated by an evolutionary distance > 0.30, which can be divided further into subtypes separated by that of 0.15. Three individuals possessed two different TTV variants of distinct genotypes, with predominant genotypes different between plasma and PBMC. Another possessed TTV of the same genotype in both the plasma and PBMC, but clones with a subtype not seen in plasma were observed in PBMC. A third individual had TTV variants with or without a deletion mutation, and those with the deletion mutation abounded only in PBMC. The remaining three individuals were infected with TTV with the same sequence both in plasma and PBMC. These results indicate that TTV variants with phylogenetic differences could infect the same individual, and that some variants would have a predilection for PBMC. It remains to be seen, however, if TTV replicates in PBMC or whether it has been sequestered before its evolution in the host. ***J. Med. Virol. 57:252–258, 1999.***

KEY WORDS: hepatitis viruses; genotypes; blood cells; parvoviridae infections

INTRODUCTION

Hepatitis C virus (HCV), which was discovered by Choo et al. [1989], is responsible for most cases of acute posttransfusion as well as chronic non-A, non-B hepatitis [Alter et al., 1989]. However, the exclusion of blood units contaminated with HCV and those with hepatitis B virus (HBV) has not abolished posttransfusion hepatitis [Alter and Bradley, 1995]. There are patients with cryptogenic hepatitis with or without cirrhosis in whom serological markers of HBV or HCV are not detectable [Kodali et al., 1994]. Furthermore, most patients with fulminant hepatitis are without markers of hepatitis A virus (HAV), hepatitis E virus (HEV), hepatitis D virus (HDV), HBV, or HCV [Feray et al., 1993; Fagan and Harrison, 1994].

These observations point to hepatitis virus(es) other than HAV, HBV, HCV, HDV, or HEV that would be responsible for acute and chronic hepatitis of unknown etiology. As candidates for unknown hepatitis viruses, GB virus C (GBV-C) [Simons et al., 1995] and hepatitis G virus (HGV) [Linnen et al., 1996], which is another isolate of GBV-C, have been reported independently by two groups of investigators. GBV-C/HGV is a single-stranded RNA virus of approximately 9,400 nucleotides, resembling flaviviruses in genomic organization and distantly related to HCV [Leary et al., 1996; Linnen et al., 1996]. Because these are separate isolates of the same virus, they will be referred to collectively as GBV-C/HGV for convenience.

However, there is no evidence for the replication of GBV-C/HGV in the liver [Laskus et al., 1997], nor is there any proof for its hepatitis-inducing capacity [Alter, 1997; Miyakawa and Mayumi, 1997]. Hence, there

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TABLE I. Genotypes of TTV Clones From Plasma and PBMC in Eight Symptom-Free Carriers

Case no.	Plasma			PBMC		
	N	Major clones *n* (genotype)[a]	Minor clones *n* (genotype)[a]	*N*	Major clones *n* (genotype)[a]	Minor clones *n* (genotype)[a]
Group A: Different genotypes or a deletion mutation between plasma and PBMC						
1	6	5 (1a)	1 (2c)	6	6 (2c)	0
2	10	10 (1b)	0	6	4 (3a)	2 (1b)
3	6	6 (3a)	0	6	4 (2d)	2 (3a)
4	10	10 (2d)	0	6	4 (2d)	2 (2e)
5	10	9 (1a)	1 (1a)[b]	6	6 (1a)[b]	0
Group B: Same genotype between plasma and PBMC						
6	6	6 (1b)	0	6	6 (1b)	0
7	6	6 (1b)	0	6	6 (1b)	0
8	6	6 (1b)	0	6	6 (1b)	0

TTV, TT virus (non-A to G hepatitis virus); PBMC, peripheral blood mononuclear cells.
[a]Genotypes of TTV were defined by an evolutionary distance >0.30 and labeled by Arabic numbers, such as 1, 2, and 3, in the phylogenetic analysis indicated in Figure 3. Genotypes were classified further into subtypes with an evolutionary distance >0.15, and labeled by small-case letters, such as a, b, and c (Fig. 3).
[b]Deletion of 1 bp (indicated in Fig. 2) was detected in the sequence of 222 bp.

still would be other hepatitis virus(es). Recently, a novel DNA virus was identified that has no significant sequence similarity to known viruses [Nishizawa et al., 1997]. TTV develops in the circulation of some patients with posttransfusion hepatitis of unknown etiology, in close association with elevated alanine aminotransferase (ALT) levels [Nishizawa et al., 1997]. TTV is an unenveloped, noncircular and single-stranded DNA virus [Okamoto, Nishizawa et al., 1998], and as such, resembles parvoviruses amongst the known animal viruses.

DNA of TTV is detected by the polymerase chain reaction (PCR) with semi-nested primers in levels from 10- to 100-times higher in the liver than the corresponding serum from some patients with chronic non-A to G hepatitis [Okamoto, Nishizawa et al., 1998]. It was detected in 9 of the 19 (47%) patients with fulminant hepatitis and 41 of the 90 (46%) patients with chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma of unknown etiology.

TTV is prevalent in the general population of Japan, with the viral DNA detected in 34 of 290 (12%) blood donors [Okamoto, Nishizawa et al., 1998]. Such a high prevalence might reflect a nonparenteral transmission because TTV DNA is detected in feces from carriers [Okamoto, Akahane et al., 1998]; some animal parvoviruses such as feline parvovirus and mink enteritis virus are excreted into feces [Pattison, 1990]. Like human parvovirus B19 and animal parvoviruses [Pattison, 1990], TTV may have a tropism for hematopoietic cells. For the purpose of evaluating the tropism to hematopoietic cells and possible replication in such cells, TTV DNA was sought and the sequence determined in peripheral blood mononuclear cells (PBMC) from apparently healthy individuals with circulating virus in plasma.

MATERIALS AND METHODS

Subjects

Thirty apparently healthy individuals in Japan who had TTV DNA in plasma were studied. They had normal ALT levels (≤40 IU/L) in serum, and were without hepatitis B surface antigen (HBsAg) or antibody to HCV, except for one laboratory member who was a symptom-free carrier of HBsAg with antibody to hepatitis B e antigen (Case 2 in Table I). HBsAg was tested by passive hemagglutination (MyCell, Institute of Immunology Co., Ltd., Tokyo, Japan) and antibody to HCV by passive hemagglutination (Abbott HCV PHA 2nd Generation, Dainabot, Tokyo, Japan) or by enzyme-linked immunosorbent assay (ELISA-II, Ortho Diagnostic Systems, Tokyo, Japan).

Separation of Plasma and PBMC

Plasma was separated by centrifugation at 680 × *g* for 15 min at 25°C, from 10 ml of whole blood added with 50 μl of 0.5 M ethylenediamine tetraacetic acid (EDTA). Phosphate-buffered saline (Dulbecco's PBS[–], Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) was added to the precipitate to a total volume of 25 ml, and suspended. The suspension was overlaid onto 25 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 680 × *g* for 20 min at 25°C to obtain PBMC. The PBMC fraction was washed with PBS(–) three times, and suspended in 1 ml of PBS(–). The final wash did not contain detectable TTV DNA, indicating that PBMC would have been freed of TTV in the plasma.

Extraction of Nucleic Acids

Nucleic acids were extracted with phenol/chloroform from 50 μl of plasma pre-treated with proteinase K and sodium dodecyl sulfate by the method described previously [Okamoto et al., 1990]. Extracted nucleic acids were dissolved in 20 μl of Tris-HCl buffer (10 mM, pH 8.0) supplemented with 1 mM EDTA (hereafter referred to as TE buffer), heated at 95°C for 15 min and quickly chilled on ice. A half amount, equivalent to 25 μl of plasma, was tested for TTV DNA by PCR.

The PBMC suspension (200 μl), equivalent to 2 ml of whole blood, was centrifuged at 6,500 × *g* for 2 min, and the supernatant was discarded. The pellet was sus-

pended in 800 μl of DNA zol reagent (Gibco-BRL, New York) by vortexing for 1 min mechanically, and mixed with 400 μl of 100% (wt/wt) ethanol at room temperature. The mixture was centrifuged at 14,000 × *g* for 15 min at 20°C, rinsed with 75% ethanol twice, and air-dried. The sediment was dissolved in 200 μl of TE buffer, heated at 95°C for 15 min followed by immediate chilling on ice, and a 10-μl portion (equivalent to 100 μl of whole blood) was tested for TTV DNA.

Detection of TTV DNA by the Semi-Nested PCR

TTV DNA was determined by PCR with semi-nested primers compatible with different genotypes and subtypes [Okamoto, Nishizawa et al., 1998] and Perkin-Elmer AmpliTaq DNA Polymerase (Roche Molecular Systems, Inc., New Jersey). In brief, the first round of PCR was carried out with NG059 primer (sense: 5′-ACA GAC AGA GGA GAA GGC AAC ATG-3′) and NG063 (antisense: 5′-CTG GCA TTT TAC CAT TTC CAA AGT T-3′) for 35 cycles (94°C, 30 sec; 60°C, 45 sec; 72°C, 45 sec [additional 7 min for the last cycle]), and the second-round PCR with NG061 (sense: 5′-GGC AAC ATG YTR TGG ATA GAC TGG-3′ [Y = T or C; R = A or G]) and NG063 for 25 cycles with the same conditions. The amplification products by the first-round PCR measured 286 base pairs (bp), and those by the second-round PCR 271 bp.

Nucleic acids extracted from plasma (equivalent to 25 μl) and PBMC (contained in 100 μl of whole blood) were serially diluted 10-fold in TE buffer supplemented with 20 μg/ml glycogen (Boehringer Mannheim, Mannheim, Germany), and then determined for TTV DNA by PCR. The highest dilution (10^N) testing positive was determined, and used as a relative titer for estimating the distribution of TTV DNA in plasma and PBMC among the subjects.

Determination of TTV Sequences

Products of the second-round PCR were ligated to pCR2.1 vector (Invitrogen Corp., California), and using plasmid DNA extracted from transformed *Escherichia coli,* both strands were sequenced with ThermoSequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham International plc, Buckinghamshire, England).

Computer Analysis of Nucleotide Sequences

Sequence analysis of TTV isolates was undertaken using ODEN program version 1.1.1 (National Institute of Genetics, Mishima, Japan) and Genetyx-Mac version 8.0 (Software Development Co., Tokyo, Japan). The evolutionary distance was estimated by the six-parameter method [Gojobori et al., 1982]. The phylogenetic tree was constructed by the unweighted pair-group method with arithmetic mean [Nei, 1987], and also by the neighbor-joining method [Saitou and Nei, 1987].

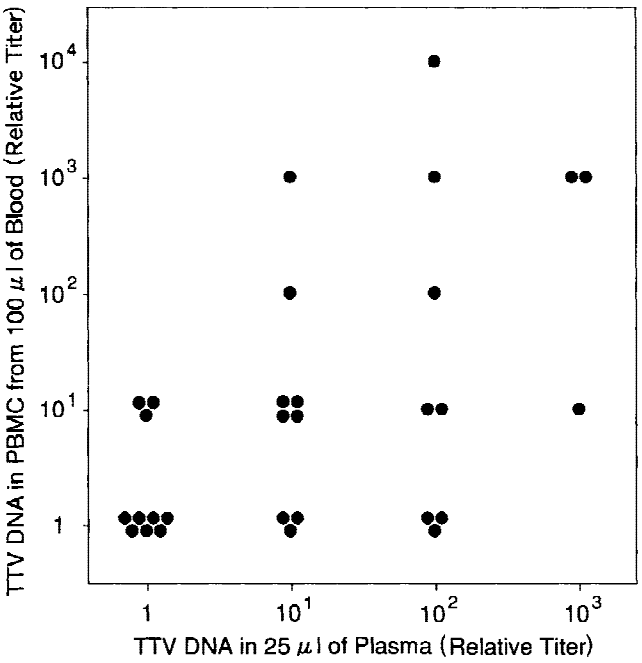

Fig. 1. Comparison of (TT Virus) DNA titers between peripheral blood mononuclear cells (PBMC) and plasma in 30 symptom-free carriers. Relative titers were determined in TTV DNA extracted from 25 μl plasma and that from PBMC contained in 100 μl whole blood for each carrier.

RESULTS

TTV DNA in PBMC From Individuals Who Possessed It in Plasma

TTV DNA was detected in PBMC from all 30 individuals with TTV in the plasma and without any clinical symptoms. Relative titers of TTV DNA were estimated in PBMC and plotted against those in plasma (Fig. 1). There was an approximate correlation (r = 0.542) of TTV DNA titers between plasma and PBMC.

Sequence Comparison of TTV DNA Clones Between PBMC and Plasma

A TTV DNA sequence of 222 bp was determined on 6–10 clones each from plasma and 6 clones each from PBMC from eight individuals selected randomly from among the 30 (Fig. 2). Genotypes and subtypes of TTV were determined by a sequence divergence ~30% and ~14%, respectively [Okamoto, Nishizawa et al., 1998]. Five possessed distinct TTV variants with major species different between PBMC and plasma (Group A in Table I). For three of these five, genotypes of major, predominant clones were different between plasma and PBMC. For Case 1, two different genotypes were identified in the TTV DNA clones from plasma (1a and 2c), whereas the minor one (2c) was observed only in those from PBMC. For Cases 2 and 3, the genotype of predominant clones in plasma was possessed only by minor clones in PBMC. Case 4 possessed TTV of the same subtype (2d) both in PBMC and plasma, but clones with a subtype (2e) not seen in plasma were observed only in PBMC.

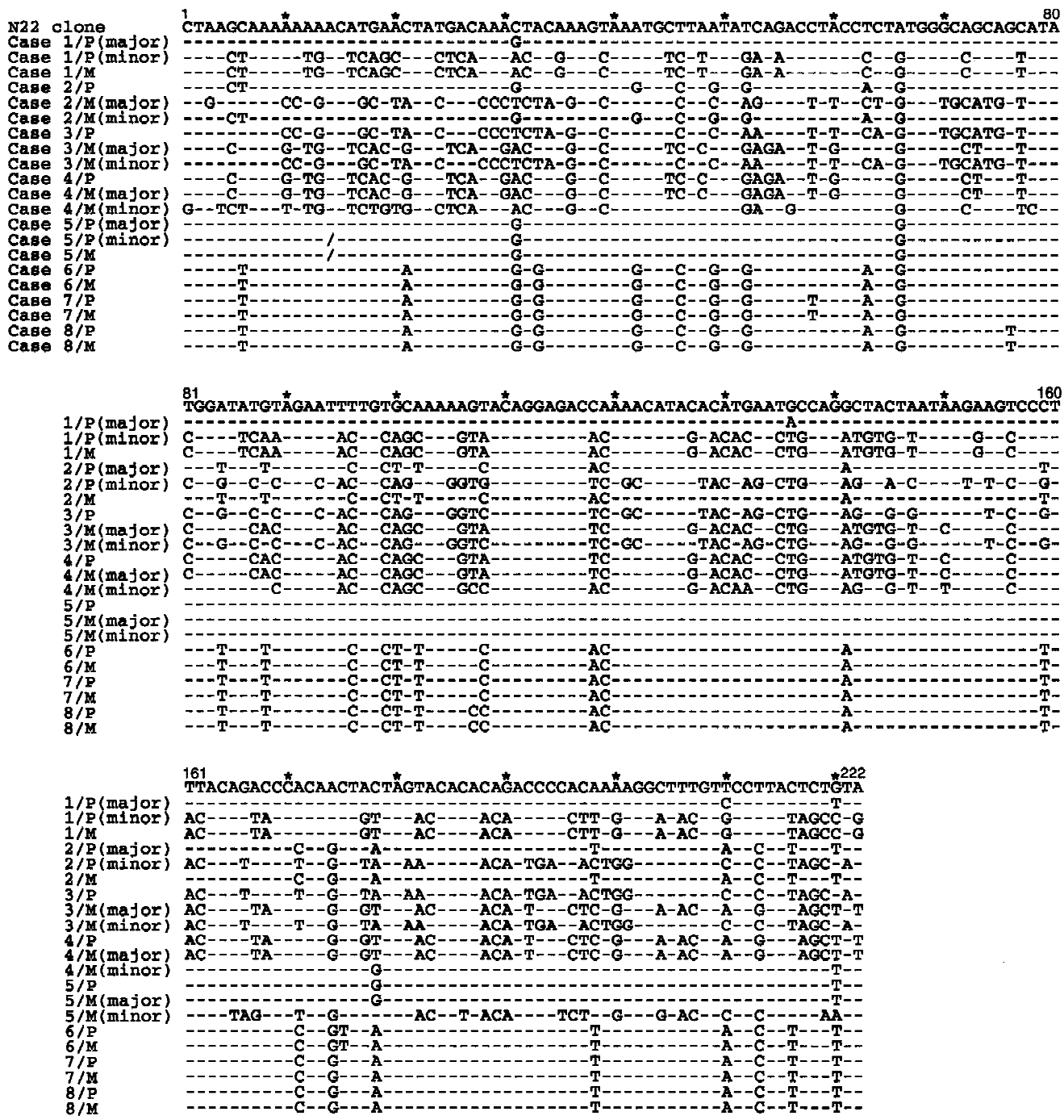

Fig. 2. Nucleotide sequences of (TT Virus) DNA from plasma and peripheral blood mononuclear cells (PBMC) from eight symptom-free carriers. The sequence of 222 bp of amplification products of the second-round polymerase chain reaction (PCR) (primer sequences at both ends excluded) is shown. Sequences of TTV DNA in plasma are indicated by P and those in PBMC by M after the slash. In cases for which two different sequences were obtained, major and minor sequences are indicated in parentheses.

Although TTV DNA clones in Case 5 had the identical genotype (1a), they differed by deletion of one nucleotide between plasma and PBMC (Fig. 2). Thus, the deletion mutation found in all six clones from PBMC was detected in only 1 (10%) of the 10 clones from plasma (Table I).

The remaining three individuals (Cases 6–8) had TTV DNA clones in plasma of the genotype (1b) identical to that of those in PBMC (Group B in Table I).

Phylogenetic Analysis of TTV DNA Clones From Plasma and PBMC

A phylogenetic analysis was carried out by the unweighted pair-group method with arithmetic mean

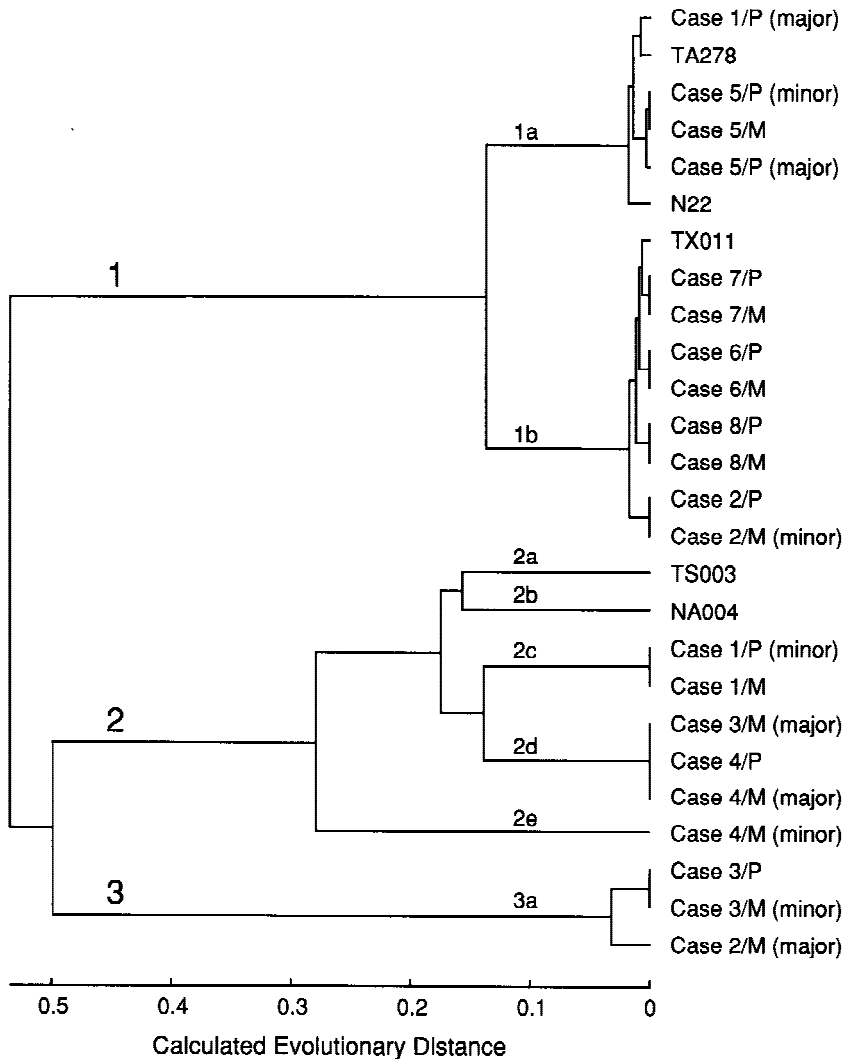

Fig. 3. A phylogenetic tree of (TT Virus) based on comparison of the 222-bp sequence among 26 isolates. P after the case number represents plasma and M represents peripheral blood mononuclear cells (PBMC) with major or minor populations indicated in parentheses. N22 represents the original TTV clone [Nishizawa et al., 1997], and TA278, TX011, TS003, and NA004 represent the reported TTV clones [Okamoto, Nishizawa et al., 1998]. Classification into genotypes (labeled by arabic numbers) was based on an evolutionary distance > 0.30 and that into subtypes (labeled by lower case letters) on a distance of > 0.15.

[Nei, 1987], within the sequence of 222 bp, on the 21 TTV DNA clones obtained from Cases 1–8 and the five reported of four distinct subtypes [Nishizawa et al., 1997; Okamoto, Nishizawa et al., 1998]. They were classified into three genotypes separated by an evolutionary distance > 0.3 (Fig. 3). Genotypes were further divided into subtypes separated by an evolutionary distance > 0.15, such as 1a and 1b as well as 2a and 2b reported previously [Okamoto, Nishizawa et al., 1998], and additional 2c, 2d and 2e. The same classification was obtained by the neighbor-joining method [Saitou and Nei, 1987], also.

When the 21 TTV sequences were subjected to a 2 × 2 comparison, along with the five previously reported [Okamoto, Nishizawa et al., 1998], those in distinct genotypes differed by 33.3–43.2%, those of distinct subtypes in the same genotype by 11.3–26.6%, and those in the same subtype by 0–3.2%.

DISCUSSION

Bradley et al. [1983] found two distinct non-A, non-B hepatitis viral agents by chimpanzee transmission studies. One agent was sensitive to chloroform whereas the other was resistant. The agent sensitive to chloroform, and presumably enveloped, was identified as HCV in a later study [Bradley et al., 1990]. The other, resistant to chloroform and supposed to be nonenveloped, has not been identified as yet, and has been described briefly as hepatitis X virus [Murphy, 1996]. Nonenveloped virus-like particles with a diameter of 22 nm, resembling parvoviruses, have been observed by immune electron microscopy in sera and feces of marmosets infected experimentally with GB or Berlin agent implicated in non-A, non-B hepatitis in human beings [Almeida et al., 1976; Appleton, 1977].

It is not clear how TTV is related to these viral

agents, which induce hepatitis in primates. A notable characteristic of TTV is that it is detected frequently in sera from apparently healthy individuals. TTV DNA was detected in 34 (12%) of 290 blood donors in Japan, although the prevalence was much higher in patients with non-A to G fulminant or chronic hepatitis at 47 and 46%, respectively [Okamoto, Nishizawa et al., 1998]. TTV is excreted into feces from carriers for a possible, fecal-oral transmission [Okamoto, Akahane et al., 1998]. Although, human parvovirus B19 is not excreted in feces, many animal parvoviruses are shed into stools [Berns, 1996]. The transmission of TTV both parenterally, typically by transfusions [Okamoto, Nishizawa et al., 1998], and nonparenterally by a fecal-oral route would promote its spread in the community.

TTV appears to infect and replicate in the liver, because the titers of TTV DNA in liver biopsies were from 10- to 100-times higher than those in the corresponding sera [Okamoto, Nishizawa et al., 1998]. In addition, TTV may infect hematopoietic cells. In the present study, TTV DNA was detected in PBMC from all 30 individuals in Japan with circulating virus in the plasma. B19 parvovirus is extraordinarily tropic for human erythroid cells, cultures of human bone marrow, peripheral blood, and a few leukemic cells or cell lines [Young, 1996]. Animal parvoviruses such as porcine parvovirus, feline panleukopenia virus, and a variant of the minute virus of mice can infect lymphocytes [Pattison, 1990]. Taken together, TTV might be able to infect hematopoietic cells, as is the case with human and animal parvoviruses.

However, the detection of TTV DNA in PBMC gives rise to more questions than answers. First, detection of viral DNA in PBMC may not indicate the infection and replication of TTV there. TTV could simply adhere to PBMC and be engulfed without replication. Secondly, subpopulations of PBMC harboring TTV are not known. It needs to be determined if T or B lymphocytes, or both, contain TTV. Thirdly, the state of TTV in terms of either episomal or chromosomal would have to be determined. Adeno-associated viruses, in the genus *Dependovirus* of the *Parvoviridae,* are known to be integrated into cellular DNA in latent infection [Berns, 1996].

TTV has a wide range of sequence divergence, which occurs in two tiers as for HCV [Okamoto, Nishizawa et al., 1998]. The hierarchical distribution of sequence divergence has been confirmed in the present study with an additional range of diversity. The divergence in a higher order with an evolutionary distance > 0.30 will be attributed to different genotypes as has been proposed for HCV by Simmonds et al. [1993], and labeled by arabic numbers such as 1, 2, and 3. Likewise, the divergence in a lower order with an evolutionary distance > 0.15 will be attributed to distinct subtypes of the same genotype and distinguished by lower case letters, such as a, b, and c. In a previous report [Okamoto, Nishizawa et al., 1998], two genotypes, 1 and 2, which break down further into subtypes, 1a and 1b as well as 2a and 2b, were recognized. In the present study, a new genotype, named 3, and new subtypes in the genotype 2, such as 2c, 2d, and 2e, were identified. It is remarkable that such a wide variation of TTV has been found in surveys restricted to Japan.

Additional new genotypes and subtypes may well be detected in TTV isolates in unexamined areas in the world, as has been demonstrated by HCV. Aside from such an epidemiological value, genotypes and subtypes of TTV might be associated with a particular form of disease or response to treatment as is the case with HCV [Bukh et al., 1995; Miyakawa et al., 1995; Simmonds, 1995]. Although TTV has not been sequenced entirely as yet, such variants of TTV would need to be characterized by the entire genomic sequences as has been achieved for HCV [Okamoto and Mishiro, 1994] and GBV-C/HGV [Okamoto et al., 1997].

Of particular note are different genotypes and subtypes of TTV variants detected between PBMC and plasma (Table I). This finding invites a possibility of different organ tropisms for particular TTV variants, as has been shown with parvoviruses [Maxwell et al., 1995; Spitzer et al., 1997; Ueno et al., 1997], as well as HCV [Shimizu et al., 1997; Navas et al., 1998] and human immunodeficiency virus (HIV) [Epstein et al., 1991]. In all these studies, however, a tropism for different tissues was ascribed to only one or a few amino acid changes in the *env* (capsid for unenveloped viruses) or *pol* protein, or both.

Phylogenetic differences of TTV variants between PBMC and plasma, with different genotypes and subtypes separated by an evolutionary distance > 0.30 and > 0.15, respectively, resemble phylogenetically distinct HIV variants infecting different organs of the body, such as brain, spleen, and lymph nodes [Wong et al., 1997]. Such a wide divergence among viral strains infecting different organs of the same individuals may reflect anatomically distinct, independent evolution of quasispecies named as "virodemes" after the independent evolution of members of a species that are separated by geography [Wong et al., 1997]. It is equally possible, however, that TTV of a certain genotype was simply compartmentalized in PBMC, and has escaped immune pressure irrespective of the evolution of circulating (and presumably hepatic) virions. It would be worthwhile to follow TTV genotypes both in PBMC and plasma in the same individuals. Also, the replication of TTV and integration of the viral DNA in PBMC need to be determined.

The mutation rate is not known for the TTV genome. It is not hard to conceive, however, that it would take years or even decades to evolve beyond the threshold of genotypes. Because it is unlikely that the majority of PBMC can survive this period and keep episomal infection, TTV DNA may well be integrated into chromosomes for the clonal replication of a certain genotype. Some parvoviruses, known as dependoviruses, are integrated into chromosomes [Berns, 1996]. Another possibility is that carriers had been co-infected with TTV strains with different genotypes and organ tropisms either at the outset or as a result of super-infection.

Further studies are required to determine genotype-dependent organ tropism of TTV, replication in organs other than the liver, and the strategy to infect hosts persistently.

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